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Evaluation of Five New Plating Media for Isolation of *Salmonella* Species

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A three-phase study was conducted to compare Hektoen enteric agar (HE), Rambach agar (Ra), SM-ID medium (SM), xylose-lysine-Tergitol 4 agar (XLT4), novobiocin-brilliant green-glycerol-lactose agar (NBGL), and modified semisolid Rappaport-Vassiliadis medium (MSRV) for the recovery of nontyphoid salmonellae from stool specimens. After evaluation of the first two phases, which resulted in the elimination of Ra, SM, and NBGL, 593 consecutive stool samples were investigated by plating them directly and after tetrathionate enrichment at 37°C on HE, XLT4, and MSRV. A total of 82 *Salmonella*-positive stool specimens were detected (positivity rate, 13.8%). Sensitivities for direct plating and after tetrathionate enrichment were 32.9 and 86.6%, respectively, for XLT4, 63.4 and 100.0%, respectively, for MSRV, and 34.1 and 79.3%, respectively, for HE. Specificities (percentage of morphologically suspicious colonies that were indeed salmonellae) were 100.0 and 99.8%, respectively, for XLT4, 99.0 and 98.8%, respectively, for MSRV, and 67.9 and 75.0%, respectively, for HE. The use of MSRV instead of HE increased the isolation rate of salmonellae by 26.2% (65 versus 82 strains isolated from HE and MSRV, respectively). We conclude that MSRV is the most sensitive medium tested and is a very specific medium for the isolation of nontyphoid salmonellae from stool specimens. However, its semisolid nature is a disadvantage and requires careful handling in the laboratory, especially when salmonellae are present. XLT4 had a sensitivity comparable to that of HE and a nearly 100% specificity and can be regarded as an alternative for the isolation of nontyphoid salmonellae from stool samples.

Five selective media for the isolation of *Salmonella* spp. have recently been described: Rambach agar (Ra; E. Merck, Darmstadt, Germany) (16), SM-ID medium (SM; bioMériéux S.A., Montalieu-Vercieu, France) (15), xylose-lysine-Tergitol 4 agar (XLT4; Difco Laboratories, Detroit, Mich.) (12), novobiocin-brilliant green-glycerol-lactose agar (NBGL; not commercially available) (14), and modified semisolid Rappaport Vassiliadis medium (MSRV; Difco) (1, 3, 4, 7, 10, 13). In comparison with established media, these formulations promise facilitated recovery of salmonellae because of either higher sensitivity (percentage of *Salmonella*-positive stools that yield *Salmonella* spp. on a particular medium) or higher specificity (percentage of plates with colonies resembling *Salmonella* spp. from which salmonellae are isolated).

Screening of stool samples for the presence of salmonellae on Hektoen enteric Agar (HE) (11) is labor-intensive because of the high number of colonies (e.g., *Proteus* spp. and *Citrobacter* spp.) that resemble *Salmonella* spp. This results in a low specificity and, consequently, in additional costs for subsequent identification. Ra and SM contain moderate amounts of bile salts to inhibit coliforms. True *Salmonella* spp. appear as red colonies because of acid formation from propylene glycol for Ra or D-glucuronate for SM, whereas colonies of coliforms either become blue, green, or violet if they are positive for β-galactosidase activity (cleavage of a chromogenic substrate) or remain colorless (5, 15, 16). XLT4 utilizes the detergent Tergitol 4 to obtain nearly complete inhibition of *Proteus* spp., and *Salmonella* colonies develop a black color because of H_2S formation from sodium thiosulfate, which is visualized by the incorporated ferric ammonium citrate (12). In NBGL, sufficient H_2S formation is only achieved by colonies that do not produce acid from glycerol or lactose, because a low pH interferes with H_2S formation. This results in colorless colonies for most *Proteus* and *Citrobacter* species, in contrast to the black colonies of *Salmonella* species (14). MSRV is based on the swarming phenomenon of motile bacteria (*Salmonella* spp. and others) at reduced agar concentrations. Coliforms are inhibited by a combination of increased osmotic pressure, malachite green, and incubation at 41 to 43°C. Several isolated drops of liquid stool (or stool liquefied by the addition of 0.9% saline) are inoculated onto the surfaces of the plates. Ra, XLT4, MSRV, and NBGL are not suitable for use in the isolation of typhoid *Salmonella* serotypes (*Salmonella typhi* or *Salmonella paratyphi* type A); only SM detects salmonellae of such serotypes.

In order to evaluate the five new media we compared them with HE (BBL Becton-Dickinson, Basel, Switzerland), our standard medium for the isolation of salmonellae and shigellae. We divided our study into three phases, as follows. (i) Thirty-two stool specimens known to contain salmonellae and 32 specimens without salmonellae were analyzed by direct plating on all six media. In addition, samples were enriched both in tetrathionate and in selenite broth at 37 and 42°C, respectively, and were then subcultured onto all media except MSRV. (ii) One hundred seventy-seven consecutive stool specimens were enriched in tetrathionate at 37°C (this was followed by subculturing on HE, XLT4, MSRV, and SM) and/or at 42°C (this was followed by subculturing on HE and Ra). NBGL was omitted at this stage because of poor performance in phase 1. (iii) Five hundred ninety-three consecutive stool samples were analyzed with HE, XLT4, and MSRV by direct plating as well as after tetrathionate enrichment at 37°C.

MATERIALS AND METHODS

HE, Ra, and MSRV are available as commercial products and were prepared according to the manufacturers' instructions. SM was obtained as commercially prepared plates. XLT4 was prepared from two different commercial lots. Into lot

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1 (used in phases 1 and 2) 4.6 mg of Tergitol 4 were already incorporated, and 1.2 g of proteose peptone no. 3 (Difco) per liter had to be added separately. Lot 2 (used in phase 3) required the separate addition of 4.6 ml of Tergitol 4 (Sigma 139-88-8) per liter, but no additional proteose peptone was required. This change in formulation was done by Difco because of the reduced shelf-life when Tergitol 4 is incorporated (4a). Lot 2 represented the final formulation that was released for sale by Difco. NBGL is not available commercially and was prepared with 40.0 g of Trypticase soy agar (BBL 11043), 1.5 g of ferric ammonium nitrate (Merck 3762), 5 g of sodium thiosulfate (Merck 6518), 10.0 g of lactose, 10 ml of glycerol (Merck 4094), 7 mg of brilliant green (Fluka 16020), 10 mg of novobiocin, and 1,000 ml of distilled water (14). All media were prepared in batches of 5 liters each. The ingredients were poured into plates (20 ml), and the plates were stored open at 4°C and were used within 3 weeks. Each lot of media was tested after preparation with strains of *Escherichia coli*, *Salmonella enteritidis*, and *Salmonella typhimurium* as internal quality controls. Stool samples were received in liquid Cary Blair transport medium (2), but without agar. They were processed continuously upon arrival in the laboratory. One drop of liquid stool (or stool liquefied by the addition of an appropriate volume of 0.9% NaCl) was used to inoculate the solid media by using a disposable plastic pipette, and the inoculum was streaked with a glass stick and was fractionated by using a second glass stick. Twelve individual drops of stool were inoculated in a circle close to the periphery of a semisolid MSRV agar plate. Enrichment broths (10 ml) were inoculated with 1 ml of stool, incubated overnight at 37 and 42°C, and streaked onto solid media as described above. For inoculation of MSRV from enrichments, only 6 to 8 drops instead of 12 drops were used since higher counts of salmonellae were expected.

Media inoculated directly from stool suspensions were inspected for growth of suspicious colonies after 15 to 19 h of incubation, whereas media inoculated from enrichment broths were examined after 24 h of incubation. Inspection of directly inoculated MSRV was repeated after a total incubation of 24 h. Rose-colored colonies on Ra were first tested for their oxidase activities (on pieces of filter paper impregnated with *N,N,N,N*-tetramethyl-1,4-phenylendiamine-dihydrochloride). Only oxidase-negative colonies were processed further, since in our experience the presence of slightly rose-colored colonies of *Pseudomonas* spp. reduced the specificity of this medium. Suspect colonies from solid media or swarming growth from MSRV were streaked to a quarter of a triple sugar iron (TSI) agar plate (BBL), and one drop (50 μl) of phage O-1 suspension (Biokema, Crissier-Lausanne, Switzerland) (6) was placed in the center of the streaked area. After incubation for 6 h at 37°C the plates were inspected for visible phage lysis. Positive colonies were confirmed by slide agglutination tests with O antisera (O polyvalent antiserum and individual group A, B, C_1, C_2, D, and E antisera; Behringwerke AG, Marburg, Germany) and by an in-house biochemical identification system that comprised tests (in glass tubes) for DNase, lysine and ornithine decarboxylase activities, indole production, motility, production of H_2S, gas formation from glucose, acid formation from lactose or sucrose (TSI tube), acid from adonitol, and malonate utilization (17).

RESULTS

Phase 1. In direct plating, Ra and SM were slightly less sensitive than HE, thus confirming our previous report (5) and the findings of Heizmann (8, 9), whereas XLT4 and MSRV were more sensitive than HE (Table 1). NBGL showed the worst sensitivity in direct plating and after enrichment, especially when enrichment was done at 37°C. All other media performed well when they were inoculated with samples from enrichments, with sensitivities of 97% and greater. The specificities of the media varied between 84 and 100% for direct plating and between 69 and 100% after enrichment (Table 2).

Phase 2. The aim of the second phase was a more thorough investigation of the performances of the media after enrichment. Enrichment at 42°C was found to increase both sensitivity (Table 1) and specificity (Table 2). Although the sensitivities of SM and Ra approached that of HE, XLT4 and MSRV showed still higher sensitivities than those of the first three media. The specificity of XLT4 was outstanding: virtually every deep black colony on this medium was a *Salmonella* sp. Slide agglutination of *Salmonella* O antigens directly from such colonies can be done easily. High degrees of specificity were also achieved with MSRV, Ra, and HE, but for the latter only when it was inoculated with samples from an enrichment at 42°C instead of 37°C.

Phase 3. On the basis of the results presented above, XLT4 and MSRV were then further evaluated and compared with HE, our standard medium. We inoculated 593 consecutive stool samples onto HE, XLT4, and MSRV directly as well as following tetrathionate enrichment overnight at 37°C. Eighty-two stool samples were positive for salmonellae on one of the media. The sensitivity of MSRV was considerably increased when incubation was extended to a full 24-h period (Table 1), a fact that had already been observed by Aspinall et al. (1). MSRV plates incubated for a full 24-h period reached a level of sensitivity that was achieved by HE or XLT4 only after enrichment. Extending the incubation time considerably be-

TABLE 1. Sensitivities of five media in detecting *Salmonella* strains

Step and medium	Sensitivity (%)		
	Phase 1 (P = 32)[a]	Phase 2 (P = 28)	Phase 3 (P = 82)
Direct plating			
HE	75	39	34
XLT4	88	ND[b]	33
MSRV	100	ND	63,[c] 85[d]
RA	69	ND	ND
SM	59	ND	ND
NBGL	53	ND	ND
After enrichment			
HE	97,[e,f] 100[g,h]	82,[g] 89[h]	79[g]
XLT4	97,[e,f,g] 100[h]	89[g]	87[g]
MSRV	ND	96[g]	100[g]
RA	97,[e,f] 100[g,h]	82[h]	ND
SM	97,[e,f] 100[g,h]	83[h]	ND
NBGL	81,[g] 97[h] 88,[e] 94[f]	ND	ND

[a] P, number of samples positive for salmonellae.
[b] ND, not done.
[c] Incubation for 15 to 19 h.
[d] Incubation for 24 h.
[e] Selenite at 37°C.
[f] Selenite at 42°C.
[g] Tetrathionate at 37°C.
[h] Tetrathionate at 42°C.

TABLE 2. Specificities of five media

Step and medium	Specificity (%)[a]		
	Phase 1 (*n* = 32)	Phase 2 (*n* = 149)	Phase 3 (*n* = 511)
Direct plating			
HE	84	74	68
XLT4	100	ND[b]	100
MSRV	ND	ND	97,[c] 99[d]
RA	91	ND	ND
SM	84	ND	ND
NBGL	100	ND	ND
After enrichment			
HE	69,[e] 81,[f] 72,[g] 75[h]	70,[e] 87[f]	75[e]
XLT4	100[e,f,g,h]	100[e]	99.8[e]
MSRV	ND	96[e]	99[e]
RA	97,[e,g,h] 100[f]	91[f]	ND
SM	88,[g] 100,[f] 91,[e] 97[h]	83[e]	ND
NBGL	94,[e] 100[f,g,h]	ND	ND

[a] *n*, number of negative specimens.
[b] ND, not done.
[c] Incubation for 24 h.
[d] Incubation for 15 to 19 h.
[e] Tetrathionate at 37°C.
[f] Tetrathionate at 42°C.
[g] Selenite at 37°C.
[h] Selenite at 42°C.

yond 24 h, however, resulted in an increase in the number of false-positive results (mainly *Enterobacter* sp. and *Citrobacter* sp. [data not shown]). Again, XLT4 impressed us by its outstanding specificity: we found only one false-positive plate from 511 enriched negative stool samples (Table 2). In direct plating there were no false-positive colonies. However, when compared with the results of the first two phases, the sensitivity of XLT4 on direct plating seemed to be decreased. This may be related to the change in the formulation of the medium (inclusion of proteose peptone but the separate addition of Tergitol 4), although the exact reason for the difference is not obvious.

The distributions of the O serotypes among the isolated salmonellae were as follows: group B, $n = 11$; group C_1, $n = 15$; group C_2, $n = 5$; group D, $n = 52$. One stool sample yielded two different serotypes (B and C_1); therefore, 83 strains were isolated from 82 positive samples. *S. typhi* or *S. paratyphi* type A was never found, whereas two of the serotype B isolates belonged to *S. paratyphi* type B and were easily detected on each of the three media. Other organisms that were isolated from our 593 stool samples were *Campylobacter jejuni* or *Campylobacter coli* ($n = 32$ [5.4%]), *Shigella* spp. ($n = 8$ [1.35%]), *Aeromonas* spp. ($n = 4$ [0.67%]), and *Yersinia enterocolitica* ($n = 2$ [0.34%]; serotypes of *Y. enterocolitica* not known).

DISCUSSION

The poor performance of NBGL in the first phase is in contradiction to the original description of Poisson (14). We felt that NBGL in many instances failed to produce sufficient blackening of typical *Salmonella* colonies; therefore, although salmonellae were present, they were not recognized. In general, there was a smaller number of false-positive colonies from salmonella-negative stool samples when enrichments were incubated at 42°C than when they were incubated at 37°C. However, in a pilot test with stock cultures of *S. paratyphi* type A, we found that *S. paratyphi* type A colonies were not recovered on either medium when enrichments were incubated at 42°C (two strains tested). Therefore, the higher incubation temperature, although more effective for the isolation of nontyphoid *Salmonella* serotypes, should not be applied when the occurrence of *S. paratyphi* type A must be considered, and that is why we chose 37°C for the incubation of enrichments in the third phase of our study.

MSRV, XLT4, and Ra are not suitable for use in the isolation of *S. typhi* and *S. paratyphi* type A. Disease caused by typhoid *Salmonella* serotypes usually starts with a high fever in the absence of diarrhea, and therefore, the causative organisms will usually be found in blood cultures rather than stool samples. During the 5-year period from 1989 through 1993, 21 patients infected with *S. typhi*, 5 infected with *S. paratyphi* type A, 4 infected with *S. paratyphi* type B, and none infected with *S. paratyphi* type C were identified. Patients infected with *S. paratyphi* type B are not considered here further since in our study organisms of this serotype were easily isolated from all of our media. Of the remaining 26 patients, 17 had positive blood cultures for either *S. typhi* ($n = 13$) or *S. paratyphi* type A ($n = 4$), and 11 of them never had positive stool samples at any time during the course of their disease. On the other hand, for nine patients the diagnosis was established by stool culture alone. We would have failed to correctly diagnose these nine patients in a 5-year period if one of the media unable to detect typhoid serotypes had been used exclusively. However, of these nine patients, eight had a history of traveling abroad and one was designated a "salmonella carrier" on the examination form. Therefore, we decided to include HE in our protocol in addition to MSRV for the testing of stool specimens from all patients with a history of traveling abroad or who were designated salmonella carriers. SM, however, would be a reasonable alternative, since its sensitivities after enrichment were similar to those of HE.

We conclude that XLT4 and especially the semisolid MSRV, which requires careful handling, are excellent media for use in the recovery of nontyphoid salmonellae from stool specimens. They are not only more sensitive than HE but also more specific and, therefore, reduce the workload in the diagnostic laboratory considerably. For the isolation of *S. typhi* and *S. paratyphi* type A, however, an additional medium is required (HE or SM).

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